

## Cigarette Smoking Is Associated With Increased Human Monocyte Adhesion to Endothelial Cells: Reversibility With Oral L-Arginine but Not Vitamin C

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**Objectives.** This study sought to assess the effect of cigarette smoking on adhesion of human monocytes to human endothelial cells and to measure the effect of L-arginine and vitamin C supplementation on this interaction.

**Background.** Cigarette smoking has been associated with abnormal endothelial function and increased leukocyte adhesion to endothelium, both key early events in atherogenesis. Supplementation with both oral L-arginine (the physiologic substrate for nitric oxide) and vitamin C (an aqueous phase antioxidant) may improve endothelial function; however, their benefit in cigarette smokers is not known.

**Methods.** Serum was collected from eight smokers (mean  $\pm$  SD age  $33 \pm 5$  years) with no other coronary risk factors and eight age- and gender-matched lifelong nonsmokers. The serum was added to confluent monolayers of human umbilical vein endothelial cells and incubated for 24 h. Human monocytes obtained by counterflow centrifugation elutriation were then added to these monolayers for 1 h, and adhesion then was measured by light microscopy. To assess reversibility, monocyte/endothelial cell adhesion was then measured for each subject 2 h after 2 g of oral vitamin C and 2 h after 7 g of oral L-arginine.

**Results.** In smokers compared with control subjects, monocyte/endothelial cell adhesion was increased ( $46.4 \pm 4.5\%$  vs.  $27.0 \pm 5.2\%$ ,  $p < 0.001$ ), endothelial expression of intercellular adhesion molecule (ICAM)-1 was increased ( $0.31 \pm 0.02$  vs.  $0.22 \pm 0.03$ ,  $p = 0.004$ ), and vitamin C levels were reduced ( $33.7 \pm 24.1$  vs.  $53.4 \pm 11.5$   $\mu\text{mol/liter}$ ,  $p = 0.028$ ). After oral L-arginine, monocyte/endothelial cell adhesion was reduced in smokers (from  $46.4 \pm 4.5\%$  to  $35.1 \pm 4.0\%$ ,  $p = 0.002$ ), as was endothelial cell expression of ICAM-1 (from  $0.31 \pm 0.02$  to  $0.27 \pm 0.01$ ,  $p = 0.001$ ). After vitamin C, there was no significant change in monocyte/endothelial cell adhesion or ICAM-1 expression from baseline in the smokers despite an increase in vitamin C levels (to  $115 \pm 7$   $\mu\text{mol/liter}$ ).

**Conclusions.** Cigarette smoking is associated with increased monocyte-endothelial cell adhesion when endothelial cells are exposed to serum from healthy young adults. This abnormality is acutely reversible by oral L-arginine but not by vitamin C.

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Cigarette smoking is a major risk factor for the development of atherosclerosis, with smokers having at least a 2.5 fold increase in the incidence of coronary heart disease compared with nonsmokers (1). Cigarette smoke has been associated with abnormal endothelial function in animal models of atherosclerosis (2) and in young adult active and passive smokers (3,4). Cigarette smoke also increases leukocyte adhesion to endothelial cells (5,6), an important early event in atherogenesis, and is

associated with a reduction in plasma levels of vitamin C (7). Because vitamin C is an excellent aqueous phase antioxidant in human plasma (8), and has been shown in animal models to slow the progression of atherosclerosis (9) and reduce leukocyte aggregation (10), it has been suggested by some investigators (8) that supplementation with vitamin C may be protective in smokers.

In cholesterol-fed rabbits, L-arginine, the physiologic precursor for nitric oxide, improves endothelial dysfunction, decreases monocyte adhesion to endothelial cells and reduces atheroma formation (11-14). It has recently been shown that oral and parenteral L-arginine improve endothelial function in hypercholesterolemic humans (15-17) and may reduce platelet aggregation in healthy young men (18). However, the effect of L-arginine on endothelial cell/monocyte interactions in smokers has not been studied. We therefore assessed the effects of vitamin C and L-arginine supplementation on the interaction between highly purified populations of human monocytes and

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**Abbreviations and Acronyms**

EDTA	= ethylenediaminetetraacetic acid
ELISA	= enzyme-linked immunosorbent assay
ICAM-1	= intercellular adhesion molecule-1
HBSS	= Hank's balanced salt solution
HUVECs	= human umbilical vein endothelial cells
IL-1 <sub>beta</sub>	= interleukin-1 <sub>beta</sub>
IL-6	= interleukin-6
RPMI	= Roswell Park Memorial Institute
TNF-alpha	= tumor necrosis factor-alpha
VCAM-1	= vascular cell adhesion molecule-1

human vascular endothelial cells exposed to serum from smokers and nonsmokers.

## Methods

**Study design.** Eight current smokers (lifetime dose  $\geq 5$  pack-years) and eight age- and gender-matched lifelong nonsmokers were studied. No subject had a history of hyperlipidemia or diabetes mellitus or family history of premature coronary artery disease. All had normal blood pressure, and the nonsmokers had no history of regular passive smoke exposure. None of the subjects were taking regular medications, and all were clinically well. Each subject was seen on two occasions, separated by at least 14 days. Smokers and their matched control subjects were seen simultaneously and their sera analyzed side by side. For each blood test, smokers were requested to attend 10 to 30 min after their last cigarette and were only recruited if their smoking habits were unlikely to change over the course of the study. On each study day, subjects had a medical and smoking history taken, blood was drawn for baseline measurements, and either 2 g of vitamin C (Blackmores Ltd., Balgowlah, Australia) or 7 g of L-arginine (Scientific Hospital Supplies, Liverpool, United Kingdom) was administered orally. Two hours after each dose, blood was taken for analysis of treatment effect. This protocol was approved by the local ethics committee, and all subjects gave informed consent.

At each visit, blood was collected and stored on ice in heparinized tubes for biochemical studies (lipid measurements, vitamin C, arginine, citrulline and cotinine levels) and procoagulant-containing tubes (SST gel and clot activator vacutainer, Becton Dickinson) for serum collection, immediately centrifuged (3,000 rpm for 10 min) at 4°C and stored at -80°C until use.

**Biochemical studies.** Total cholesterol and triglyceride levels were measured enzymatically using a Hitachi 747 autoanalyzer; high density lipoprotein levels were determined after dextran sulfate magnesium precipitation; and low density lipoprotein cholesterol levels were determined using the method described by Friedwald et al. (19) (in no subject was the serum triglyceride level  $>300$  mg/dl). Arginine and vitamin C levels were measured after deproteinizing media with 2% sulfur salicylic acid using high performance liquid chromatog-

raphy. Plasma cotinine levels were measured using a rapid gas-liquid chromatographic method with a nitrogen phosphorus detector (20).

Serum levels of inflammatory cytokines were also assessed at baseline, after vitamin C and after L-arginine. Commercially available enzyme-linked immunosorbent assays (ELISA) were used to measure interleukin (IL)-1<sub>beta</sub> and interleukin (IL)-6 (Boehringer Mannheim) and to measure tumor necrosis factor (TNF)-alpha (Genzyme Corporation).

**Endothelial cell culture.** Human umbilical vein endothelial cells were harvested enzymatically, using a type II collagenase under sterile conditions, as described by Minter et al. (21), and established as primary cell cultures in M199 (Trace Biosciences, Australia) containing 20% heat inactivated human serum, L-glutamine 2 mmol/liter (ICN Biomedicals), 0.5% endothelial cell growth promoter (Starrate products, Bethungra, New South Wales), penicillin 100 U/ml and streptomycin 0.1 mg/ml. All media were prepared using endotoxin-free water (Baxter, Lane Cove, Australia) and filtered using Zeta-pore filters (Cuno Life Sciences Division). Endotoxin-free plasticware and glassware were used in all experiments.

For experimental studies, confluent human umbilical vein endothelial cell monolayers (passages 1 to 4) were trypsinized and replated on gelatin-coated 24-mm diameter tissue culture plates for monocyte adhesion studies and gelatin-coated 96-well plates (Falcon, Becton Dickinson) for studies of cell adhesion molecule expression. Plates were gelatin coated by adding 1 ml/5 cm<sup>2</sup> of Haemaccel (Behringwerke AG, Marburg, Germany) diluted 1:250 in phosphate-buffered saline, incubating for 1 h at 37°C and then decanting the solution before use. Human umbilical vein endothelial cells were grown to confluence and used within 72 h. Before use, each monolayer was inspected microscopically to ensure that only endothelial cells were present, and the purity of the cultures was periodically confirmed by staining cells using a monoclonal antibody specific for von Willebrand factor.

**Isolation of human monocytes.** White cell concentrate (Red Cross Blood Bank, Sydney, Australia) was obtained from the peripheral blood of human volunteers who had no clinical evidence of cardiovascular disease. Monocytes were isolated within 24 h of collection by density gradient separation of white cell concentrate anticoagulated with 0.07% ethylenediaminetetraacetic acid (EDTA) (Merck Pty Ltd., Kilsyth, Australia) on Lymphoprep (Nycomed Pharma, Oslo, Norway) at 20°C followed by counterflow centrifugation elutriation, as described by Garner et al. (22). A Beckman J2-21M/E centrifuge was used equipped with a JE-6B elutriation rotor and a standard 4.2-ml elutriation chamber (Beckman Instruments, Inc.). The elutriation buffer was Hank's balanced salt solution (HBSS) without calcium or magnesium (Trace Biosciences, Australia), with EDTA (0.1 g/liter) and 1% heat inactivated human serum added. The mononuclear cell fraction taken from the Lymphoprep density gradient at the Lymphoprep-plasma interface was loaded at 9 ml/min into the elutriation rotor (2,020 rpm at 20°C). The flow rate was increased by 1 ml/min every 10 min, and monocytes were eluted between 16

and 17 ml/min. Monocyte suspensions were used only if purity was >90% on light microscopy, with <1% contamination by neutrophils, and viability >95% by trypan blue exclusion. Monocytes were then resuspended in Roswell Park Memorial Institute (RPMI) medium containing 10% heat inactivated human serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml), stored in Teflon containers at a concentration between  $1.5$  and  $2.0 \times 10^6$ /ml at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  in air and used within 3 h.

**Monocyte-endothelial adhesion studies.** Confluent endothelial monolayers were incubated with M199 medium containing 50% serum from each subject under each condition for 24 h before adhesion assays. At the end of the 24-h incubation, cell viability was >95% (by trypan blue exclusion) for each condition. Experiments were repeated on at least two occasions, with four separate wells used for each condition in each subject.

Before this study, preliminary experiments were conducted to assess the effect of varying time after elutriation and varying incubation periods on monocyte adhesion to cultured endothelial cells. These studies were carried out three times on three separate weeks using 12 wells for each condition. Intraobserver error was also assessed in these initial studies, with repeated measurements taken on separate wells under the same conditions, and repeated measurements taken on the same wells, separated by time. Basal monocyte adhesion to human umbilical vein endothelial cells (HUVECs) was not significantly different at 1 h (38%), 24 h (35%) or 48 h (35%) after elutriation. Monocyte adhesion to HUVECs was maximal after 1-h incubation, increasing from 30 min (22%) to 1 h (38%) and remaining unchanged at 2 h (38%). This technique had a low intraobserver error, with a coefficient of variation <5%.

The adhesion assay was performed by adding 1 ml of monocyte suspension (in RPMI with 50% human serum from the condition being tested) at a concentration of  $1.0$  to  $1.5 \times 10^6$ /ml to each endothelial cell monolayer and incubating for 1 h at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  in air. After 1 h, nonadherent cells were removed by standardized gentle washing using a 1,000- $\mu\text{l}$  automatic pipette (Gilson), and the suspension was stored on ice until the cell concentration was counted using a Neubauer hemocytometer (Weber scientific, Middlesex, United Kingdom). The initial suspensions and the suspensions from each well were counted four times. The percentage of adherent monocytes was then calculated by comparison with the initial monocyte concentration.

**Cell adhesion molecule detection on endothelial monolayers.** Endothelial cell surface expression of adhesion molecules was assessed using an ELISA method. Confluent endothelial monolayers were preincubated for 24 h in 96-well plates for each subject, as previously described. After washing the monolayers with HBSS, monoclonal antibodies to intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule-1, E-selectin (all Becton Dickinson) and isotype mouse immunoglobulin G (ICN Immunobiologicals) (0.1  $\mu\text{g}$  in 100  $\mu\text{l}$  of HBSS with 10% heat inactivated human serum) were added and allowed to stand for 30 min. The monolayers were then

**Table 1.** Baseline Characteristics\*

	Smokers (mean $\pm$ SD)	Nonsmokers (mean $\pm$ SD)	p Value
Age (yr)	33 $\pm$ 5	31 $\pm$ 4	0.44
Cigarettes/day	26 $\pm$ 7	0.0	< 0.001
Pack-yr	14 $\pm$ 4	0.0	< 0.001
Cotinine (nmol/liter)	2,211 $\pm$ 1,092	33 $\pm$ 92	< 0.001
Total cholesterol (mg/dl)	178.9 $\pm$ 19.5	198.4 $\pm$ 31.1	0.21
Triglycerides (mg/dl)	87.3 $\pm$ 35.3	88.2 $\pm$ 26.5	0.90
LDL cholesterol (mg/dl)	112.8 $\pm$ 19.5	132.3 $\pm$ 35.0	0.15
HDL cholesterol (mg/dl)	50.6 $\pm$ 11.7	54.5 $\pm$ 15.6	0.70
Vitamin C ( $\mu\text{mol/liter}$ )	33.7 $\pm$ 24.1	53.4 $\pm$ 11.5	0.028
L-Arginine ( $\mu\text{mol/liter}$ )	128.5 $\pm$ 30.0	147.7 $\pm$ 43.1	0.33

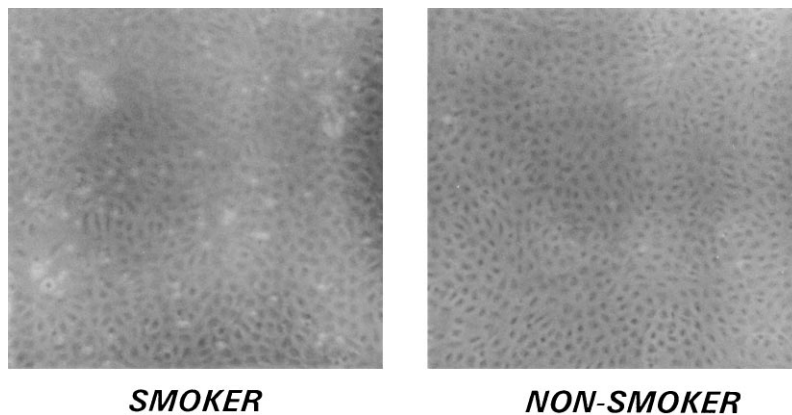
\*Groups are compared using independent sample *t* tests with Hochberg's modification of the Bonferroni procedure (see Methods). HDL = high density lipoprotein; LDL = low density lipoprotein.

washed, and sheep anti-mouse-horse radish peroxidase conjugate (Amersham International, Amersham, UK) (1:500 in 100  $\mu\text{l}$  of HBSS with 10% heat inactivated human serum and 0.05% Tween 20) was added and left for 30 min. After further washing, 150  $\mu\text{l}$  of ABTS [2,2'-azino-di(3-ethylbenzothiazoline sulfonate)] substrate (Kirkegaard and Perry Laboratories) was added to each well and allowed to develop for 15 min. Optical density was measured at 414 nm with an ELISA reader (Titertek multiscan, Flow Laboratories, Meckenheim, Finland).

**Statistics.** Data were analyzed with SPSS for Windows 6.0. All descriptive data are expressed as mean value  $\pm$  SD. Differences between smokers and nonsmokers were assessed using independent sample *t* tests, adjusted for multiple comparisons using Hochberg's modification of the Bonferroni procedure (23). Differences between baseline, vitamin C and L-arginine were assessed using repeated measures analysis of variance, followed by the Scheffé procedure for multiple comparisons. Statistical significance was inferred at a two-tailed *p* value < 0.05.

## Results

The baseline characteristics of the subjects are summarized in Table 1. Smokers had a higher level of plasma cotinine than nonsmokers (*p* < 0.001). Cotinine levels in the smokers were similar on each blood collection, consistent with a stable pattern of cigarette exposure. Vitamin C levels were significantly reduced in smokers compared with nonsmokers ( $33.7 \pm 24.1$  versus  $53.4 \pm 11.5$   $\mu\text{mol/liter}$ , *p* = 0.028). Other baseline characteristics, such as age and lipid and L-arginine levels, were not different between the two groups. Both vitamin C and L-arginine supplementation were well tolerated, although one subject complained of a mild headache after L-arginine administration. After vitamin C administration, serum vitamin C levels rose significantly in both smokers (from  $33.7 \pm 24.1$  to  $115.8 \pm 40.3$   $\mu\text{mol/liter}$ , *p* < 0.001) and nonsmokers (from  $52.4 \pm 11.5$  to  $133.4 \pm 47.5$   $\mu\text{mol/liter}$ , *p* < 0.001). Vitamin C levels rose to at least normal levels in all subjects after



**Figure 1.** Photomicrographs showing monocyte adhesion to HUVEC monolayers, randomly taken from the center of 25-mm wells. The monolayer on the **left** (smoker) has been incubated for 24 h with media containing 50% serum from a smoker before the adhesion study. The monolayer on the **right** (non-smoker) has been incubated for 24 h in media containing 50% serum taken from a nonsmoker.

supplementation (range 66.0 to 190.0  $\mu\text{mol/liter}$  in smokers and 58.0 to 176.0  $\mu\text{mol/liter}$  in nonsmokers). After L-arginine, arginine levels rose significantly in both smokers (from  $128.5 \pm 29.9$  to  $294.9 \pm 63.9$   $\mu\text{mol/liter}$ ,  $p = 0.005$ ) and in nonsmokers (from  $147.7 \pm 43.1$  to  $235.7 \pm 25.0$   $\mu\text{mol/liter}$ ,  $p < 0.01$ ). Citrulline levels were similar in both groups at baseline ( $26.0 \pm 7.1$   $\mu\text{mol/liter}$  in smokers,  $23.1 \pm 2.2$   $\mu\text{mol/liter}$  in nonsmokers) and did not change with L-arginine supplementation.

There was no difference in serum cytokine levels at baseline between smokers and nonsmokers (IL-1 $_{\beta}$ :  $25.6 \pm 19.9$  pg/ml in nonsmokers,  $32.7 \pm 15.2$  pg/ml in smokers,  $p = 0.13$ ; IL-6:  $10.4 \pm 6.6$  pg/ml in nonsmokers,  $12.75 \pm 5.9$  pg/ml in smokers,  $p = 0.45$ ; TNF- $\alpha$ :  $27.8 \pm 18.3$  pg/ml in nonsmokers, and  $33.8 \pm 17.4$  pg/ml in smokers,  $p = 0.5$ ). None of the measured cytokines changed significantly in smokers or nonsmokers after either vitamin C or L-arginine (data not shown).

**Cigarette smoking is associated with increased monocyte adhesion to endothelial cells.** Monocyte adhesion to endothelial cells exposed to serum from smokers was significantly higher than adhesion to endothelial cells exposed to serum from nonsmokers ( $46.4 \pm 4.5\%$  vs.  $27.0 \pm 5.2\%$ ,  $p < 0.001$ ), as illustrated in Figure 1.

**Increased monocyte adhesion to endothelial cells is associated with increased surface expression of ICAM-1.** Surface expression of ICAM-1 on human umbilical vein endothelial cells was higher in cell monolayers exposed to serum from smokers than in those from nonsmokers (optical density:  $0.31 \pm 0.02$  vs.  $0.22 \pm 0.03$ ,  $p = 0.004$ ) (Table 2). However, no difference was seen in the surface expression of vascular cell

adhesion molecule (VCAM)-1 (optical density:  $0.13 \pm 0.02$  in smokers vs.  $0.11 \pm 0.01$  in nonsmokers,  $p = \text{NS}$ ) or E-selectin (optical density:  $0.19 \pm 0.03$  in smokers vs.  $0.20 \pm 0.01$  in nonsmokers,  $p = \text{NS}$ ).

**L-Arginine but not vitamin C supplementation reduces monocyte adhesion to endothelial cells.** Monocyte adhesion to endothelial cells exposed to serum from smokers was reduced when the endothelial cells were exposed to serum taken from the same smokers after oral L-arginine supplementation (from  $46.4 \pm 4.1\%$  to  $35.1 \pm 4.0\%$ ,  $p = 0.002$ ) (Fig. 2). However, no change was seen in nonsmokers after L-arginine supplementation ( $26.9 \pm 5.1\%$  to  $27.6 \pm 5.5\%$ ,  $p = \text{NS}$ ). After vitamin C supplementation, there was no significant change in the degree of monocyte adhesion to endothelial cell monolayers in either smokers ( $46.4 \pm 4.5\%$  to  $41.9 \pm 8.5\%$ ,  $p = \text{NS}$ ) or nonsmokers ( $27.0 \pm 5.2\%$  to  $26.8 \pm 5.6\%$ ,  $p = \text{NS}$ ).

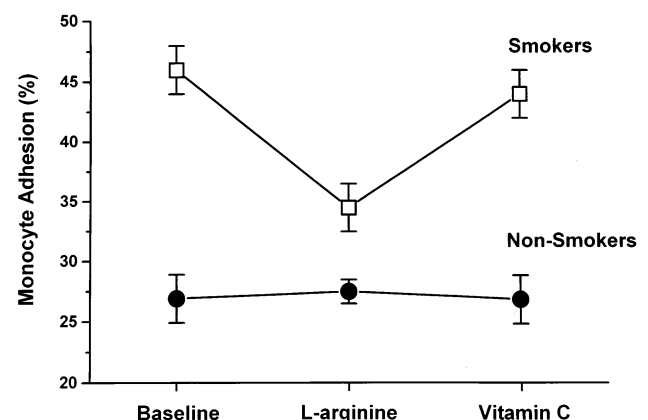
There was no associated change in the surface expression of either VCAM-1 or E-selectin after exposure of the endothelial monolayers to serum from subjects after vitamin C or

**Table 2.** Baseline Cellular Differences Between Smokers and Nonsmokers

	Smokers (mean $\pm$ SD)	Nonsmokers (mean $\pm$ SD)	p Value
Monocyte adhesion (%)	$46.4 \pm 4.4$	$26.9 \pm 5.1$	0.001
ICAM-1 (optical density)	$0.31 \pm 0.02$	$0.22 \pm 0.03$	0.004
VCAM-1 (optical density)	$0.13 \pm 0.02$	$0.11 \pm 0.01$	0.21
E-selectin (optical density)	$0.19 \pm 0.03$	$0.20 \pm 0.01$	0.64

\*Groups are compared using independent sample  $t$  tests with Hochberg's modification of the Bonferroni procedure (see Methods). ICAM-1 = intercellular adhesion molecule-1; VCAM-1 = vascular cell adhesion molecule-1.

**Figure 2.** Effect of L-arginine and vitamin C on monocyte adhesion to human umbilical vein endothelial cell monolayers. **Error bars** represent standard error of the mean. Levels of monocyte adhesion in nonsmokers are lower than those in smokers at baseline ( $p = 0.001$ ). The level of monocyte adhesion to endothelial cells in smokers was reduced after L-arginine administration ( $p = 0.002$ ) but not after vitamin C ( $p = \text{NS}$ ). All groups were compared by repeated measures analysis of variance, followed by the Scheffe test for multiple comparisons.



L-arginine. However, there was a small reduction in ICAM-1 expression in smokers after L-arginine supplementation (from  $0.31 \pm 0.02$  to  $0.27 \pm 0.01$ ,  $p = 0.001$ ), consistent with the reduction seen in monocyte adhesion to endothelial cells.

## Discussion

Monocyte adhesion to endothelium is a key early event in atherogenesis, preceding monocyte entry into the subintima, transformation into macrophages, lipid loading and fatty streak formation (24). Even in the first decades of life, cigarette smokers exhibit greater fatty streak and atheroma formation than nonsmokers (25). In the present study, we showed that cigarette smoking is strongly associated with increased monocyte adhesion to endothelium and that serum from smokers induces greater endothelial cell expression of ICAM-1 than that from control subjects. Furthermore, the smoke-related increase in monocyte/endothelial adhesion was almost completely reversed by the administration of a single oral dose of L-arginine, the physiologic precursor of nitric oxide (26) and associated with decreased surface expression of ICAM-1. In contrast, a single dose of vitamin C did not affect the degree of adhesion, in this model.

**Smoking and atherosclerosis.** The mechanism of accelerated atherogenesis in relation to smoking is unknown; however, both animal (27) and human studies (28) have confirmed a continuous and graded relation between smoke exposure and atherosclerosis. Cigarette smoke contains thousands of chemical compounds (29) that may affect both the arterial wall or circulating blood components, thus increasing the risk of clinical cardiovascular disease. For example, smokers have abnormal platelet aggregation (30), abnormalities of circulating blood leukocytes (6,31), increased fibrinogen levels (32) and enhanced thrombin generation (33). All of these may contribute to both atherosclerosis and thrombosis.

**Smoking, endothelium and nitric oxide.** Tobacco has a direct toxic effect on human endothelium (34,35). Such injury to the arterial wall may be particularly important in precipitating the cellular events of atherogenesis (24). In vitro, it has been shown that serum from smokers is directly cytotoxic to cultured endothelial cells (36). In vivo, we previously showed that endothelium-dependent dilation, a nitric oxide-dependent phenomenon (37), is markedly impaired in young adult smokers (3). It is also known that endothelial release of nitric oxide is important in the control of intercellular adhesion molecule expression, through a nuclear factor- $\kappa$ B-dependent pathway (38). In the current study, inflammatory cytokine levels were not different in smokers and nonsmokers, suggesting that these changes in endothelial function may not be mediated by alterations in cytokine levels. Similarly, Anderson et al. (39) previously reported changes in neutrophil activity of passive smokers that was not associated with alterations in cytokine levels. Although the regulation of cell adhesion molecules is complex, the cigarette smoke-related enhancement of monocyte/endothelial adhesion in this study may be due (at least in part) to impaired endothelial nitric oxide release, with conse-

quent increased expression of intercellular adhesion molecules. In this context, it is interesting that a single oral dose of L-arginine, the nitric oxide precursor, was able to decrease significantly the monocyte adhesion to endothelium in smokers, and that this was associated with decreased endothelial expression of ICAM-1.

L-Arginine has previously been shown to have important antiatherogenic effects, both in animals (11-14) and in humans (15-18). In hypercholesterolemic rabbits, oral L-arginine may reduce atheroma formation (11), decrease cholesterol-related endothelial dysfunction, reduce platelet aggregation and decrease monocyte adhesion to aortic endothelium (11-14). In humans, both acute parenteral and longer term oral therapy with L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults (15,16), and oral L-arginine has recently been shown (18) to inhibit platelet aggregation in healthy young men through the nitric oxide pathway. There have been no previous reports of the effects of L-arginine in smokers or of L-arginine's effects on monocyte/endothelial interactions in humans. However, L-arginine does appear to improve endothelium-dependent dilation in smoke-exposed, cholesterol-fed rabbits (40). The mechanism of the beneficial effect of L-arginine in smokers may be related to enhanced endothelial production of nitric oxide, an antioxidant effect of L-arginine, which may decrease nitric oxide catabolism by smoke-enhanced oxygen-derived free radicals (41), or other mechanisms.

**Smoking, monocytes and vitamin C.** The important interaction between monocytes and the endothelium is complex and may also be affected by monocytes as well as a variety of biochemical factors (42). It has been shown (10) in animal models, using an intravital microscopic technique, that cigarette smoke increases leukocyte aggregation and adhesion to endothelial cells in vivo. In vitro studies of human cells have also documented this increase in monocyte/endothelial cell adhesion (6). Kalra et al. (5) observed changes in both circulating monocytes and in cultured endothelial cells when exposed to cigarette smoke; in particular, they noted an increase in the expression of CD11b ligand on the surface of monocytes as well as an increase in endothelial expression of ICAM-1 and E-selectin. Weber et al. (31) recently demonstrated an enhanced expression of the integrin CD11b/CD18 on monocytes isolated from smokers and that this was associated with increased adhesiveness to cultured endothelial cells. Other circulating factors such as platelet activating factor-like lipids may also play a role in modifying the monocyte/endothelial cell interaction (43). Due to the complexity of monocyte/endothelial interactions, interventions aimed at reducing atherogenesis in cigarette smokers may need to address smoke-induced changes in both monocyte as well as endothelial factors.

Vitamin C has been proposed by some investigators as a potentially protective agent in smokers due to its antioxidant properties (8), particularly because cigarette smoke is an important source of circulating reactive oxygen intermediates (44). Vitamin C levels are significantly decreased in smokers

(7,45). In the study by Weber et al. (31), 10 days of vitamin C reversed the smoke-related increase in monocyte expression of CD11b/CD18 and returned monocyte adhesion to unstimulated endothelial cells toward normal (although there was no effect on adhesion to stimulated endothelial cells, as are usually found at sites of atherosclerosis) (46). This is consistent with some *in vitro* work, where vitamin C has been shown (10) to reduce leukocyte/endothelial adhesion during oxidized low density lipoprotein exposure and *in vivo* animal studies demonstrating reduced leukocyte adhesion with acute parenteral administration of vitamin C. However, in our model, oral vitamin C consistently failed to influence human monocyte to human endothelial cell adhesion in either smokers or non-smokers, despite an increase in serum vitamin C levels to >50 to 60  $\mu\text{mol/liter}$  in all subjects (which is above the estimated threshold for effective protection from cardiovascular disease) (47). It may be that prolonged elevation in vitamin C levels may be required for benefit in humans rather than simply high circulating vitamin C levels in the blood stream, although a single oral dose of vitamin C may improve endothelial function in patients with coronary artery disease (48). Possibly, the optimal strategy for normalizing monocyte/endothelial adhesion in smokers involves decreasing endothelial as well as monocyte adhesiveness, for example, by a combination of L-arginine and vitamin C.

**Study limitations.** Although our subjects were well matched for age, gender and the absence of other vascular risk factors apart from smoking, it is possible that there were unmeasured differences between the smokers and nonsmokers that might account for the observed increase in monocyte/endothelial adhesion seen in the smokers. Because monocyte/endothelial adhesion would be very difficult to study *in vivo* in humans, we used a model of monocyte/endothelial cell adhesion that is accurate and reproducible and utilizes highly purified populations of human cells only. Despite this, the *ex vivo* model used in this and other (31) studies may not accurately reflect the *in vivo* situation. For example, human venous endothelial cells do not usually express adhesion molecules; the endothelial cells in our model did express such molecules, which may actually be analogous to the situation in arterial endothelial cells *in vivo* (49). It is also possible that some of the components of cigarette smoke in human serum may have been altered or metabolized during the 24-h incubation times in culture, especially because many are labile (29); despite this, our results are consistent with other models, where increased monocyte/endothelial adhesion has consistently been found in association with cigarette smoking. Other *ex vivo* experiments (31) have used isolated monocytes from smokers, rather than using serum, to study monocyte/endothelial adhesion. Because human serum is complex and contains factors that may both increase and decrease monocyte/endothelial cell adhesion (50), the use of isolated human cells without serum may not reflect the *in vivo* situation. For example, the presence of soluble cell adhesion molecules may alter monocyte/endothelial cell interactions. For this reason, we utilized a model where complete serum from each subject is incubated with endothelial cells

before monocyte adhesion was assessed. The model used in our study primarily assesses the effect of L-arginine and vitamin C on endothelial adhesiveness and does not look at possible differences between the monocytes of individual subjects or the effect of vitamin C and L-arginine on monocyte physiology.

**Clinical implications.** Because monocyte adhesion to endothelial cells is a key early event in atherogenesis and appears to be enhanced in young healthy adult smokers, this emphasizes that smoking *at an early age* can damage the arterial wall. This is consistent with both the physiologic evidence of endothelial damage that we previously observed in young smokers (3,4) and with the fatty streak formation seen even in teenage smokers (25). Intervention aimed at an early stage of the atherogenic process maximizes the chances of reversibility of arterial disease (51). Clearly, prevention of smoking by young adults would be the optimal strategy, or cessation in those who already smoke (although it is not established that smoking cessation would reverse the enhanced monocyte/endothelial adhesion observed in this and other studies). However, where these strategies are not successful, intervention with L-arginine may be a promising therapy because it is a naturally occurring substance with an excellent safety profile (18). Nevertheless, long-term efficacy needs to be proved. On the basis of work to date, a synergistic beneficial effect with oral vitamin C may also be possible.

**Conclusions.** Cigarette smoking is associated with increased monocyte adhesion to endothelial cells, a key early event in atherosclerosis. This is associated with increased expression of ICAM-1 and decreased plasma levels of vitamin C. In our study, oral L-arginine but not vitamin C supplementation was associated with a reduction of monocyte/endothelial cell adhesion in smokers. Whether L-arginine will prove useful as a protective agent in smokers requires further prospective evaluation.

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